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CHEMOTROPISM IN RHIZOPUS NIGRICANS[1]

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(WITH FOUR FIGURES)

Historical

In view of the important relation of the question of chemotropism among the fungi to the problems of fungal parasitism, it is surprising that investigations in this field have been so meager; for while numerous scattered observations may be found here and there in the literature, the works of MIYOSHI (8) and FULTON (5) stand out as the only ones which represent any considerable investigations of the subject. The researches of CLARK (3), although intended primarily to supplement his work on the toxicity of copper compounds, are also of especial importance in this connection.

It is needless to enter here into a detailed historical account of all the researches from which information can be culled regarding chemotropism in the fungi, for an excellent review of these may be found in FULTON's paper. It is our purpose merely to outline the results obtained by MIYOSHI, CLARK, and FULTON.

MIYOSHI (8) tested a great variety of chemical substances, and maintained that in many cases they exerted a very marked attraction on the fungal hyphae, which then grew toward them (positive chemotropism); others showed a repellent effect, so that the hyphae grew diametrically away from the diffusion center (negative

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chemotropism); while still others produced no effect. Sugars were especially attractive to the molds.

The most important methods employed by MIYOSHI were as follows: (1) the substances to be tested were injected in solution into leaves, which were then sown with spores; or (2) the substances were incorporated in a gelatin layer, on which was laid a perforated mica plate or membrane, the spores being sown above, either on the free surface of the plate or membrane, or in a superimposed layer of medium lacking the substance to be tested; or (3) fine glass capillary tubes holding the substance to be tested were introduced into a preparation containing germ tubes of the fungus growing in water or in a very dilute nutrient solution.

Where the germ tubes showed a marked turning toward the stomata, perforations, or open ends of the capillary tubes, as the case might be, and thus toward the diffusion centers of the chemical substances, the turnings were interpreted as instances of positive chemotropism. Moreover, there appeared to be an optimum concentration, beyond which such substances exerted a less and less attractive and at length a repellent effect. On the other hand, below the optimum, with gradually decreasing concentrations, the attractive effect also gradually decreased, ultimately to zero. Acids, alkalis, and some salts always produced a repellent effect, however, even in weak solutions.

Perhaps MIYOSHI's strongest point in support of his view was his statement that when no special chemical substances were offered, as, for example, when leaves were injected with pure water only, no turning resulted.

When we survey the investigations of FULTON (5) and CLARK (3), we find that, taken in combination, they completely contradict these results. CLARK injected leaves with various concentrations of copper and cobalt salts, etc., and found that in this case the germ tubes near the stomata curved toward and grew directly into them; but he obtained a similar result with leaves injected with pure water only. In experiments in which he used perforated mica plates, he found that the germ tubes near the perforation always "*grew toward the opening if it communicated with a layer in which no spores had been placed.*" Although he did not

follow out this line of work, he advanced the hypothesis that *Rhizopus* is "markedly chemotactic to some secretion of its own mycelium, and this negative chemotropism is much greater than any positive chemotropism it may have for food substances or oxygen."

The work of FULTON showed a decided advance in that, by introducing numerical methods of estimating the direction assumed by the hyphae, he placed on a numerical basis what had heretofore been more or less a matter of personal judgment. As PORODKO (9) has pointed out, in his work on chemotropism in roots, quantitative methods in this field must replace the qualitative ones hitherto used, if definite results are to be obtained. In startling contrast to MIYOSHI's results, FULTON failed to find "the existence of any definite chemotropic sensibility to nutrient substances or other chemical compounds in solution." He believed that if positive chemotropism in the fungi exists, it is not as marked as turning caused by other stimuli. He states: "All of the fungi tested show a tendency to turn from a region in which hyphae of the same kind are growing, toward one destitute of hyphae, or in which the hyphae are less abundant. . . . . This may be regarded as a negative reaction to stimuli from chemical substances which owe their origin in some way to the growing fungus."

These results were so much at variance with MIYOSHI's conclusions that they have been quoted with great reserve by such writers as BARNES (4), PRINGSHEIM (10), and JOST (6); and, on account of their conflicting nature, a restudy of the whole matter seemed imperative. The present work was undertaken, therefore, in the hope of deciding between the views of MIYOSHI on the one hand and those of FULTON and CLARK on the other.

Material and methods

Although the main part of the work was carried on chiefly with *Rhizopus nigricans* Ehrenb., preliminary tests were made also with *Botrytis cinerea* Pers. and a *Penicillium* which, after culture in the requisite media, answered most closely to *Penicillium* no. 24 Thom. Eventually the work was confined entirely to *Rhizopus* because its large spores and hyphae make the microscopic examination of the preparation easier; the comparatively short time necessary

for its growth reduces to a minimum the dangers both of contamination and of too extensive a diffusion of the substances tested; and the spores do not cling together in large clumps as in *Penicillium*.

For the experimental work, mica was selected on account of its impermeable nature. Small plates of this were cut from large sheets and perforated with a sharp needle. The area perforated measured 24×24 mm., while at two opposite sides of this square piece small winglike projections were left, in order to rest the preparation on supports. At first, the perforations were made 2 mm. apart, 121 holes thus being punched in each plate; but after experimental chemical tests demonstrated a comparatively rapid diffusion through such plates, the number was changed to 16, the holes then being spaced 6 mm. apart. After each experiment the mica plates were boiled, first in alkali, then in acid, and, after being rinsed and boiled in 2 or 3 changes of distilled water, they were dried finally in a hot air oven.

The spores were grown in thin layers or films of medium approximately o.5 mm. thick, placed on these mica plates by a method to be described presently. For the medium, agar was used invariably, both because compared with gelatin it afforded less nourishment when used without addition to the substances to be tested, and because in the manipulation of the films, as explained later, it was found impracticable to use gelatin. A 1.5 per cent agar was used for the plain or "non-nutrient" medium; while for the nutrient a double strength solution of the required substance was mixed with an equal volume of 3 per cent agar to make a medium of desired strength containing 1.5 per cent agar.

The method finally adopted for making the films and sowing the spores was as follows. As already indicated, each preparation consisted of a perforated mica plate bearing a thin film of medium on each of its surfaces. The film on one side might contain the spores, while that on the other side would contain the chemical substances to be investigated; but various combinations were tried, as will be shown later.

For various reasons it was found desirable to make all films of approximately uniform area and thickness. An area approximately 24×24 mm., the size of the perforated area in the mica plates, was

marked out on a clean slide by means of a brush and a very small quantity of vaseline. Then on a warming table were placed a small beaker in which were water, a thermometer, and a small vial holding the agar for the films. When the agar, previously melted in hot water, had fallen to 40° or slightly below, the requisite number of drops of a suitable suspension of spores in distilled water was added. With a small pipette, marked to indicate the amount of agar solution it should contain for the making of a film, the correct quantity was drawn up, quickly dropped and spread evenly on the area marked out on the glass slide by the vaseline lines. In order to facilitate an even spreading of the agar, the slides were laid on a large glass plate fixed in an exactly horizontal position with the aid of screw supports and a spirit level. It was found that it was very essential to keep the agar agitated during the process of pipetting it from the vial; otherwise the spores would not be distributed evenly. Before the agar on the glass slide had had time to set completely, the mica plate was placed carefully upon it in such a way that the perforated area covered the film, care being taken not to press the mica down and thus force the agar up through the holes. Next, the same amount of agar of the required composition, from another vial, was dropped and spread on the surface of the mica plate up to the boundaries of the perforated area. Transverse grooves in the mica, cut for this purpose, in most cases confined the agar to the perforated area. In case no spores were needed for this upper film, several drops of distilled water had previously been added to the agar, which were equal in volume to that of the spore mixture added to the agar for the lower film. After the upper film was set, the whole preparation, consisting of the mica plate between the two films of agar, was carefully pushed off the slide with the help of a scalpel. It was on account of this last operation that it was found impracticable to use gelatin, since this adhered so closely to the slide that it was impossible to remove it without breaking.

The preparation was now rested in a horizontal position,[2] by means of the extensions at each end, on cork supports in a Petri

[2] In the earlier experiments, some of the preparations were suspended vertically by means of a thread passed through one of the holes in the mica plate. This vertical position was found to have no influence on the result.

dish, lined on all sides with filter paper moistened in distilled water. In this way the films were situated in an atmosphere practically saturated with water vapor, and each film was under exactly the same conditions as regards access of air. This last was a matter of great importance, since it was found in the earlier experiments that when the preparations were laid on a glass slide, the germination was always poorer in the film next to the glass slide, although normal germination occurred at or near the edges of the film.

To save labor, usually 2 or 3 preparations of the same kind were placed in a single Petri dish. In the preliminary experiments, these dishes had been placed directly in an incubator kept at a uniform temperature of 24–25° C. Even under these circumstances, however, it was realized that the atmospheric condition within the Petri dish might not be uniform throughout, for we know, of course, that Petri dishes so prepared eventually dry out. To obviate this, so far as possible, all the dishes (piled one above the other in a column, which rested on 2 or 3 empty dishes, or on an inverted stender dish) were stood in one of the halves of a larger Petri dish (9 in. diameter) which also was lined with several thicknesses of filter paper and contained a quantity of distilled water. The whole was then covered with a bell-jar, likewise lined throughout with filter paper, and resting in the larger Petri dish so that water was continually being drawn up by the filter paper. In this way the atmosphere within the small Petri dishes was kept as close as possible to a uniform saturation point throughout each dish.

The whole apparatus was now incubated at 24–25° C., and when the germ tubes had grown to the desired length, they were killed by dropping a little 4 per cent formalin on the preparations. They could then be kept for several days if necessary, or until it was convenient to examine them microscopically.[3] For examination under the microscope, the preparation was laid very carefully on a

[3] The nature of this entire operation, and the amount of time necessary for its completion, made perfect antiseptic precautions impracticable. All that could be done was to have the apparatus scrupulously clean and sterile. As a matter of fact, the short time requisite for the incubation of *Rhizopus*, in few cases more than 10 hours, would allow bacteria little time for development, and no contamination was ever observed in these preparations.

glass slide, and since only the low power was necessary, no cover glass was used, on account of the danger of shifting the film.

In working out the results, it was found essential to get an idea of the amount of growth which had taken place. This was done by estimating the average length of the hyphae in the preparation in question. To obtain this average, only those hyphae near the holes were considered, since in the regions midway between these holes the conditions of nourishment differed from those in the immediate proximity of the holes; especially was this true in the case of germ tubes in films made of plain agar, with a nourishing substance on the other side of the mica plate. A definite area was selected near each hole, and the lengths of all the hyphae in this area noted. In this way 3 or more holes in different parts of the preparation were considered, or until a total of 15 hyphae had been counted and averaged.

Since, as will be shown later, the number of viable spores,[4] as well as the number of hyphae present, was found to have a marked influence on the results, it was desirable for comparative results to have the spore numbers approximately equal. In order to get a suspension of spores which would produce the desired number of spores to each volume of film covered by a square millimeter of surface, the following method was used. Spore-bearing mycelium was allowed to stand in water for a few moments and was then strained through a piece of muslin, which had previously been freed of its starch by thorough boiling and washing. The spore suspension was then tested as to its concentration by the use of a micrometer eyepiece containing accurately ruled squares, and by diluting, or by centrifuging and pouring off some of the water, was brought to the desired strength.

METHOD OF ESTIMATING THE INTENSITY OF THE CHEMOTROPIC REACTION.—In work of this kind, the method of estimating the reaction is all-important, since on it depends the accuracy of the

[4] It was found that the number of viable spores present bears a definite relation to the percentage of germination. When the spores were excessively numerous no germination at all resulted. The cause of this non-germination was not worked out experimentally, but it is probably referable to the same agencies discussed later in this paper; that is, the excretion of toxic products by the spores, in this case in the pre-germination stages.

results.   For this reason it is necessary to describe in some detail the method which was elaborated.   First, for delimiting the region about the hole within which the hyphae were to be considered, the following rule was employed.   Only those hyphae growing from spores which chanced to be located in a cylinder of medium situated over the hole were taken into consideration.   This cylinder was delimited as nearly as possible by the eye, in such a way that the center of its base coincided with the center of the hole, its diameter being 3 times that of the hole.   Although the holes varied considerably in diameter (8–45 μ), it was found that the average was about 16 μ, and that, by turning the fine adjustment of the microscope a suitable number of revolutions, a thickness of medium would be traversed approximately equal to one diameter of the hole.   In all cases the hyphae coming from spores directly over the hole, that is, in an inner cylinder with the hole as its base, were neglected, owing to the difficulty in many cases of assigning a definite direction to them.   Thus the region in which the germ tubes were considered was a cylindrical zone concentric with the hole, and equal in width and height to one diameter of the hole.

It is of course clear that the whole purpose of the adoption of this arbitrary region was to secure uniformity of conditions for collecting the data.   For the most exact work, indeed, the portion of the medium chosen for examination should correspond to a hemisphere whose base coincides with the base of the above described cylinder, and thus has a radius equal to 1.5 times the diameter of the hole.   However, it is practically impossible to define the outline of such a hemisphere with any accuracy by focusing, and so a cylinder of the dimensions described was chosen as the nearest practical approximation.

The next step was to determine as accurately as possible the direction taken by the hyphae.   An imaginary straight line was projected from the center of the hole to the spore itself, in cases where the germ tube was fairly straight (fig. 1); if, however, the germ tube curved markedly, this method was modified slightly, as explained below.   This line was supposed to make angles of 45° with two other imaginary lines intersecting one another at right angles at the spore.   The 4 regions, of 90° each, thus marked out by

these last two lines, were then called A, B, C, and D, with orienta-
tions as shown in fig. 1.   The hypha was then classed as A, B,
C, or D, according to the quarter in which it grew.

If the hypha curved markedly, only its tip was considered, since
the ultimate effect of the reaction was expressed in this portion.
In this case, the point of intersection of the two lines at right
angles was located at the tip, this having been connected to the
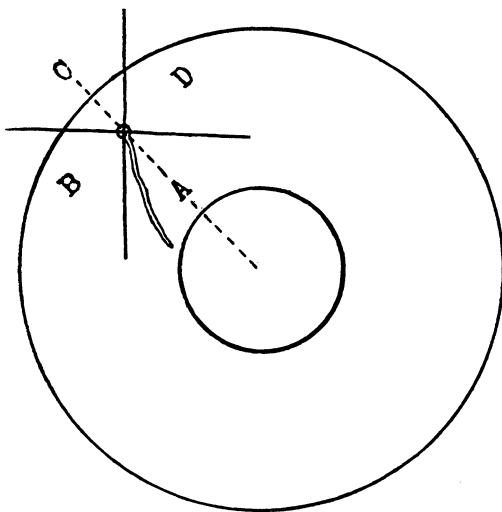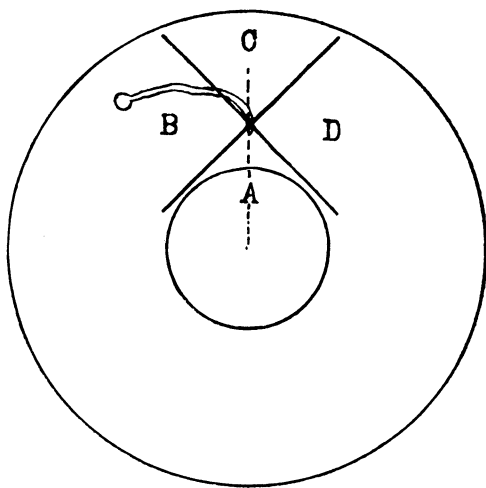
FIG. 1   FIG. 2

FIGS. 1 and 2.—Diagrams showing methods of locating directions of hyphae:
fig. 1, where the hypha is fairly straight; fig. 2, where it curves markedly.

center of the hole by a straight line, as before.   The hypha was then
classified according to the quarter into which the tip pointed (fig. 2).
In the few cases where the hyphae curved considerably up or down
from their original plane, that is, above or below an optical section
of the medium, their direction was taken as that which they would
assume if projected on their original plane.   Those growing ver-
tically up or down in the medium were neglected.

The holes in the mica plates were examined in regular succes-
sion, one row after the other, in order that no subconscious selec-
tion could be made of those that appeared more favorable to the

results expected. The data for each hole were noted down sepa-
rately, and the total was figured up when the preparation was
finished. For the most part, for the reasons already stated, 16-holed
plates were used, and usually 10 holes in each plate were examined.

The basis of the interpretation of the figures thus obtained lay
in the assumption that the average number of hyphae in each of the
4 classes, considering a large number of holes, and supposing that
no attractive or repellent forces existed, should, on the doctrine of
averages, be approximately the same, that is, 25 per cent of the
whole number; or, to put it in general terms, as many hyphae
should point in one direction as another. That this assumption
is legitimate is shown by the test count described later. Of the
whole number, then, 25 per cent would constitute the normal
number for each class, and any considerable deviation from this
would indicate a reaction to a disturbing force.

Since the *B* and *D* regions are of equal volume and equally
subjected to repellent or attractive forces, they may be left out
of consideration except in so far as the number occurring in them
goes to make up the total for each hole, and thus influences the
final percentage of reaction. In order to determine this percentage,
then, we are concerned first of all with the *A* and *C* classes. Let
us assume that an attractive force exists, derived from a substance
on the other side of the mica plate, which is causing the hyphae
to a certain extent to grow toward the holes. Since the number of
the *A* class will then be greater than the normal number, we
must conclude that those in excess of one-fourth have been attracted
into this class. If the attractive force is not a very strong one, as
indicated by a number not greatly above the normal, we may be
fairly certain that the increment has been derived from those which
would normally point right or left, that is, those of the *B* and *D*
class. The number of *A*'s in excess of the normal, however, does
not give us a complete measure of the attraction, for in all probabil-
ity some hyphae originally in the *C* class have turned into the *B* or *D*
direction, and the remaining *C*'s will therefore be fewer than one-
fourth. The difference between the remaining *C*'s and the normal
number would then represent those which have turned from the *C*
direction into *B* or *D*. By adding this number to those in excess

of the normal number in A we get the total number affected.   In order to get the total percentage of reaction, we must divide this number by three-fourths of the total number of hyphae counted in the whole area, since we are dealing with only three-fourths of the hyphae, the one-fourth represented by the normal A's being unaffected, at least visibly.[5]

If we denote the normal number to be expected in each region by n (one-fourth of the whole), the number actually in each region by its corresponding letter, and the total number of hyphae in the whole area by t, the formula will then read as follows: $\dfrac{(A-n)+(n-C)}{\frac{3}{4}t}$; or, simplifying, $\dfrac{A-C}{\frac{3}{4}t}$ = percentage of hyphae reacting.   In case a repellent force exists, raising the number of C's above the normal, the final percentage will then be a minus quantity, and it is so expressed in the accompanying tables.

One objection to this method lies in the possibility that some of the normal C's may have been attracted so strongly as to have turned from C, *through* the B or D areas, to A; these would then be counted twice over, for they would be included in the gain to A as well as in the loss from C.   It is of course quite impossible to determine for a large number of holes how many hyphae have so acted.   The objection, however, is not a serious one; for, as a matter of fact, when the number of B's and D's is fairly large, indicating a force not strong enough to draw them all into the A class, we know that the probabilities are much against the force attracting any from the C class into the A class.

However, when *more* than three-fourths of the total number point in the A direction, we can fairly assume that all which were originally B's and D's have now turned to the A direction; that is, of this 75 per cent, 25 per cent consist of those originally in A, and 50 per cent must have been derived from those nearest the A class, namely B and D; thus any number above 75 per cent must have come from those which were originally growing in the C

[5] Just what individuals among the normal A's have reacted to the stimulus we cannot of course judge, since by their original chance position they are all oriented toward the holes, but the proportion would correspond essentially to that for the remaining 75 per cent of the hyphae.

direction.   In other words, chemotropic forces which would cause a turning of slightly more than 90° would bring all the B's and D's to A; but if the force were stronger than this, some of the C class would turn to the A direction.   It is clear, therefore, that the number in excess of 75 per cent represents those which have turned from C to A; and that in this case those remaining in B and D must have been originally in the C class but have not turned strongly enough to become A's.

Accordingly, whenever the total number of hyphae in A exceeds 75 per cent of the whole number, a correction must be applied; we must now subtract from our formula the number which have come over from C into A, to avoid counting them twice.   We can ascertain, indirectly, how many of the C's have so turned into A by subtracting the number left in C from the normal number, and by also subtracting from this result the sum of those left in B or D; for, as was previously shown, those now in B and D must also have been originally C's.   The remainder, representing the difference between all these and the normal number, will then denote the number which have turned from C through the B and D classes to A.   In accordance with this, therefore, we must subtract from our first formula $[(n-C)-(B+D)]$.   The whole formula would then read: $\dfrac{(A-n)+(n-C)-[(n-C)-(B+D)]}{\frac{3}{4}t}$; or, simplifying, $\dfrac{A-n+B+D}{\frac{3}{4}t}$=percentage of hyphae reacting.   As before, should the excess over 75 per cent be in the C class, the result of the calculation would be a minus quantity, and would indicate repulsion.

THE EXPERIMENTAL ERROR.—In order to test the accuracy of the method, 10 preparations were made with conditions as regards number and length of the hyphae, and composition of the medium, essentially the same in both upper and lower films.   Under these circumstances, in accordance with our hypothesis, there should be no more turning in one direction than in another.   Hyphae were examined in the prescribed manner for 10 holes in each preparation, making 100 holes for the entire operation, or, if we count both upper and lower films, in reality 200 lots of hyphae.   The results, in the order in which the counts were made, are shown in table I.

It is evident from the results shown in table I that for small numbers, from 100 to 200, the experimental error may vary from 0 to 16 per cent; but by taking a large number, such as 774, the error is reduced to about 10 per cent. Also, apparently owing to the personal equation of the observer, the error is always minus.[6]

TABLE I

RESULTS OF TEST COUNTS TO DETERMINE ACCURACY OF METHOD

Preparation	Class				Total	Percentage of error
	A	B	C	D		
1..........	50	34	58	38	180	— 6
2..........	57	34	57	43	191	0
3..........	33	34	52	37	156	—16
4..........	24	28	39	36	127	—16
5..........	21	18	32	23	94	—16
					748	
(Average error for 748 hyphae = — 11 per cent)						
6..........	29	31	35	35	130	— 6
7..........	54	51	73	55	233	—11
8..........	41	29	49	20	139	— 8
9..........	25	28	36	33	122	—12
10..........	33	30	48	39	150	—13
					774	
(Average error for 774 hyphae = — 10 per cent)*						

* The same average error of 10–11 per cent was obtained when a recount was made after a few days' interval, so that the method can be trusted to give uniform results.

It is also noteworthy that the large error, up to 16 per cent, occurs only when the hyphae are not markedly turned in any one direction. In the results given later, where a marked turning occurs, the error would be much smaller.

FULTON'S METHOD.—In this connection, the method employed by FULTON (5) may be considered. It is stated as follows:

Hyphae within a radius of one opening diameter from the margin of each opening were considered, the hyphae within such an area were classed in the counts as those turning toward the openings, those turning away from the

[6] It is possible that the extra number in the C class may be due to some factor hitherto overlooked, which causes a slight repulsion from the holes on both sides of the plate, even when the conditions seem uniform in the two media.

openings, and those apparently indifferent. After an examination of the entire preparation in each case, those holes were selected for the counts which represented the average condition. In calculating the percentages from the counts the difference between those attracted and those repelled was made the dividend, and the total number within the observed area was made the divisor.

This method is open to at least 3 objections. In the first place, no definite method is given for distinguishing between the 3 classes of hyphae, that is, between the indifferent hyphae on the one hand, and those turning away from or toward the openings on the other hand. Without some such definite rule as that used in the method employed in the present paper, the matter of deciding their directions is likely to be influenced to a large degree by the personal equation, on account of the great variety of curves and angles which the hyphae assume. In the second place, apparently *all* the hyphae found in the area at the time of examination were included. It is clear that by such a method one must include some of the hyphae coming from spores located outside the special area; and if we are right in concluding that FULTON's classification of directions is similar to ours, it is evident that in this way a portion at least of the *A* class hyphae belonging to an outer zone would be counted, whereas the *B* and *D* classes would be mostly, and the *C* class hyphae of that zone would be entirely, neglected. Thus, even if there were no turning toward the holes at all, and the number of the *A* class in the prescribed zone were only the normal 25 per cent, by counting in some of the *A* class from the zone outside, the resulting figures would inevitably, though falsely, indicate the existence of a chemotropic reaction. Moreover, this error would be greater as the amount of real turning increased, and it would also increase with the growth in length of the hyphae, since eventually some of those from zones still more remote would reach into the prescribed zone. In the third place, although the area in the plane of the hole was clearly defined by FULTON, no statement was made as to the hyphae found in the parallel planes above the hole. If these were estimated in the same way as those in the plane of the hole, FULTON was really considering the hyphae in a cylinder of medium standing over the hole and reaching to the free surface of the film. If this were the case, he must have counted in hyphae

outside of his prescribed 1.5 diameters from the center of the hole, for in all probability his films were thicker than this.

THE RATE OF DIFFUSION THROUGH THE PERFORATED MICA PLATES.—In view of the researches of BROWN and ESCOMBE (2) on diffusion through perforated membranes, it was realized that it was essential to obtain definite data as to the rate of diffusion through the perforated mica plates. Experiments were set up in which the rates of diffusion of cane sugar through 121-holed plates and through 16-holed plates were compared for various periods of time. Without going into the details of the experiments, it may be stated that in either case, although diffusion did not proceed at such a rate as to reduce markedly the difference in concentration in the two films, yet diffusion through the 16-holed plate was naturally slower, and on this account plates so perforated were chosen for the work.

Experimental work

It is not our purpose to present a detailed account of all the experiments. In the following the most important results are outlined, using the data from representative tests. In all cases given, the results have been repeatedly verified. Altogether over 40,000 hyphae were examined and allocated to their 4 classes in the manner described above.

EXPERIMENTS WITH "STALED" SOLUTIONS.—Very early in the work, while various percentages of sugars were being tested for their power of inducing a positive chemotropism, it became quite evident that the hyphae would always turn toward the holes leading to the other agar layer, regardless of whether sugar was present in it or not, provided it contained no germinating spores. It was also significant that the more hyphae there were present in the one film, the stronger was the turning from it toward the film without spores. Furthermore, if the spores were sown in both layers, no turning resulted in either layer, or it was not nearly so pronounced. This was quite in line with the experience of FULTON and CLARK, and according to their hypothesis, previously stated, was probably due to a negative chemotropic reaction to some substance or substances excreted by the hyphae themselves. In accordance with

this theory we may thus conceive that the layer in which the hyphae were present would be permeated with their excretions, which in this paper have been termed the "staling substance or substances" (cf. BALLS 1, pp. 559, 576 ff.). Toward the region of the holes the concentration of this staling substance would be less and less, since here it is diffusing through to the other layer. This decreasing gradient of concentration would therefore act as a directive stimulus to the hyphae, causing them to grow toward the regions of less and less concentration, and thus through the holes.

In order to prove this hypothesis, the problem before us was to obtain, if possible, this staling substance free from mycelium, and determine whether, if placed in a sporeless layer, it would prevent the hyphae from growing into that layer. Although cane sugar and glucose were tried in order to obtain vigorous cultures of *Rhizopus* mycelium, and hence a strongly staled solution, the results were unsatisfactory. No medium tried gave anywhere near the vigorous growth that developed in turnip juice, prepared by pressing the juice from autoclaved white turnips.

Several experiments were performed, with unsatisfactory results, as regards a turning away from the medium containing the staled juice, the failure probably being due to the fact that the staling substances were not of the requisite strength. It was eventually found that the desired effect could be produced by allowing the juice to stale for from 3 to 4 weeks, that is, to grow the fungus in it for that length of time. For this purpose sterilized, conical flasks containing 10–15 cc. of sterile turnip juice were inoculated with *Rhizopus* and kept in an incubator at 25° C. In a few days a white weft of mycelium developed, and later sporangia appeared. By the end of 3 weeks the mycelium had taken on a brownish color, and growth had apparently ceased. The liquid remaining was now poured out of the flask. It gave an acid reaction to litmus and had a slightly sour odor of malt. Chemical tests showed the entire absence of oxalic acid or oxalates. The staled solution was now evaporated to one-half its volume, that is, to double strength, at laboratory temperature under reduced pressure. For making up the medium for the films, either one part of 6 per cent agar to 3 parts of the staled solution, or equal volumes of 3 per cent

agar and staled solution were used, in order to obtain a 1.5 per cent agar medium.

Table II gives the results of one series of experiments. In preparing the medium for this, care was taken not to heat the staled solution above 40° C.

TABLE II

NATURE OF EXPERIMENT	Mica plate →	Staled turnip juice agar +non-viable spores* / Fresh turnip juice agar +viable spores

Number of preparations examined	Period of incubation in hours	Average length of hyphae in μ	Number of spores in lower layer per sq. mm. of film surface	Direction of hyphae				Total number of hyphae counted	Percentage of reaction
				A	B	C	D		
2........	7	67	28	17	37	77	21	152	−53
4........	8.25	157	39	91	87	152	90	420	−19
2........	10	331	29	45	48	91	48	232	−26

* In this case the staled solution was not centrifuged, and this accounts for the presence of spores in this layer. They were probably dead, however, and in any case did not germinate. Their presence may or may not have had its effect on the result, but it is extremely doubtful whether they influenced it to any marked degree. Similar results were obtained in series where the solution had been freed of its spores by centrifuging.

Ten holes in each preparation were counted. As would be expected, the turning away from the holes is most marked in the youngest stage, where the hyphae have not yet produced, by their own activity, a concentration of staling substance in any way comparable to that of the other film.

In preparing these films, equal volumes of the staled turnip juice and 3 per cent distilled water agar had been used. This process diluted the staled juice to one-half strength, or, in reality, to its original strength before evaporation. One would expect, under these conditions, to see an even greater repellent effect than the results show. It is very probable, however, and is also indicated by the following experiment, that the repellent substances are of an unstable or volatile character. Although, in order to avoid any chemical change in the substance, at no time in the making of the preparations was the staled juice agar heated above 40° C., it is

possible that even this degree of heat as well as the delay due to evaporation may have led to an alteration of the staling substances.

Table III shows the results of experiments to determine whether raising the staled juice agar to the boiling point altered its repellent effect (cf. figs. 3 and 4).

TABLE III

$$\text{NATURE OF EXPERIMENT} \begin{cases} \text{Mica plate} \rightarrow \dfrac{\text{Staled turnip juice agar}}{\text{Fresh turnip juice agar}} \\ \qquad\qquad\qquad\qquad\qquad + \text{ spores} \end{cases}$$

Temperature	Number of preparations examined	Period of incubation in hours	Average length of hyphae in μ	Number of spores per sq. mm. of film surface	Total number of hyphae counted	Percentage of reaction
Staled juice not heated over 40° C.	2........ 2........	7.50 8.75	69 200	21 29	240 310	−26 −28
Staled juice heated to 100° C.	2........ 2........	7.00 8.50	57 143	31 23	168 218	+18 +72

In this series the staled solution used was 3 weeks old, and, in order to dilute it as little as possible, one part of 6 per cent agar was added to 3 parts of the staled juice, a rather difficult operation in the case where there was no heating above 40° C., owing to the fact that at this temperature 6 per cent agar is very close to its gelatinization point.

The results show that the repellent substances of the staled solution are as a whole, or in part, unstable or volatile in character (figs. 3, 4). If they had been entirely destroyed, we should get results similar to those described later for C (p. 357), that is, 100 per cent of turning. It is possible, however, that the heating entirely destroyed the repellent substances, and that the smaller reaction as compared with the preparations cited is due to the fact that most of the substances in turnip juice which induce a positive chemotropism have been used up by the fungus which originally produced the staled solution.

In this connection the work of LUTZ (7) is of interest. In the investigation of the effect of used solutions on spore germination and fungous growth, he found that in many cases high temperature

(80–100°) destroyed the effect of these solutions, or at least tempo-
rarily altered it. Furthermore, he remarks, "Wenn sich zeigen
lässt dass die uns interessierenden Stoffe durch Kochen zerstört
werden, so liegt die Annahme nahe dass es sich bei ihnen vielleicht
um fermentähnliche Körper handelt" (7, p. 106).

The theory advanced by CLARK and FULTON, therefore, that
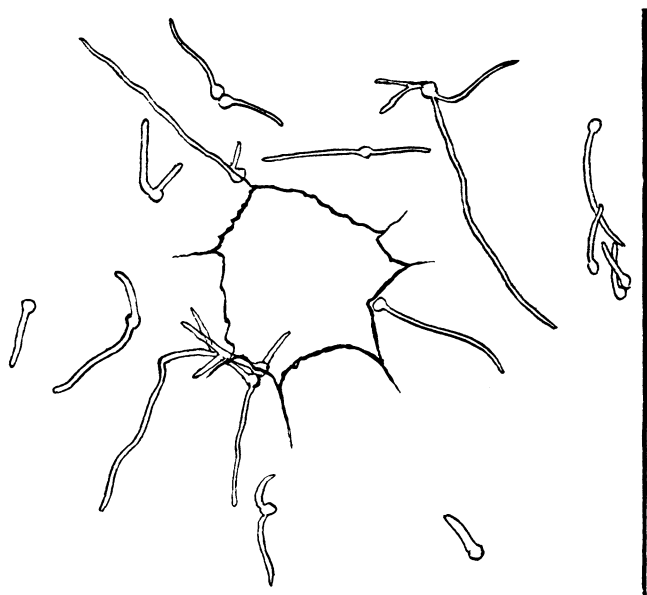during their growth the hyphae produce some substance or sub-

FIG. 3.—Germ tubes of *Rhizopus nigricans* growing away from the hole; film on
the other side of mica plate contains strong "staled" turnip juice; hyphae are growing
in a medium composed of unstaled turnip juice agar; ×80.

stances to which they are negatively chemotropic, is fully proven
by the present work, being supported by 3 lines of evidence: (1) the
hyphae turn away from a film without spores if it contains their
own staling substances; (2) the hyphae always show a marked
turning from their own medium to a second one without hyphae,
no matter what the constitution of either medium may be, unless,
indeed, the second medium contains substances which exert a
negative chemotropic stimulus; (3) when an approximately equal

amount of mycelium occurs in 2 films composed of the same medium, no turning results.

EXPERIMENTS WITH UNSTALED TURNIP JUICE.—After the existence of a negative chemotropism of this kind was proved, it became easier to search for a possible positive chemotropism. For, without going into detail, it was clear, from the fact that the hyphae are continually producing a staling substance, that the number of spores and the length of the hyphae must be considered when one is seeking to obtain evidence for positive chemotropism.

In the experiments with agar made with unstaled turnip juice, the first definite indication of a positive chemotropism (working, it is true, side by side with the negative chemotropism previously demonstrated) was obtained. The hyphae grew much more vigorously in the turnip juice medium than in the sugars. They did not in all cases grow faster; the chief difference consisted in the thickness of the germ tubes, which were twice or three times as thick as those grown in glucose or in cane sugar agar. Probably on account of this healthier development they reacted much better to chemotropic influences than when grown in the sugars. The agar was prepared by mixing equal volumes of turnip juice with 3 per cent distilled water agar, thus forming a medium containing 1.5 per cent agar. Combinations were tried as follows, the amount of reaction in each case being given below:

FIG. 4.—Germ tubes growing toward the holes; "staled" turnip juice agar has in this case previously been heated to 100° C.; otherwise conditions are the same as in fig. 3; ×80.

	A	B	C
Mica plate →	Plain agar / Turnip juice agar + spores	Turnip juice agar / Plain agar + spores	Turnip juice agar / Turnip juice agar + spores
Reaction →	+60–90 per cent	+100 per cent	+100 per cent

Since varying conditions of light, heat, and moisture had been eliminated, it is obvious that only chemotropic forces were at work here.

The turning toward the holes in preparations B and C was very marked, but in A it was not nearly so pronounced. The only difference between B and C lay in the distance from the hole at which the curvature became noticeable in the same period of time (8.5 hours). In C, the reaction was apparent at a distance of 3–4 diameters of the hole, counting from its margin; but in B, the turning could be observed as far as 10 diameters from the hole. In B and C the turning in all cases was 100 per cent, while in A it varied from 60 to 90 per cent.

In C, since the turnip juice was everywhere of practically the same concentration, the only force exerted must be due to the staling substances produced by the hyphae, that is, a negative chemotropic force. In B, however, where we have the most marked turning of all, we can fairly assert that the turnip juice in the sporeless layer is the cause of the additional stimulus, and is, therefore, a *positive* chemotropic force. This reasoning was corroborated by the condition in A, where in the lower film the positive chemotropic force, due to the turnip juice, would be working *against* the negative chemotropic force due to the staling substance, with the resulting decrease in the amount of turning.

In these experiments the hyphae had been allowed to grow to a considerable length, none being under 300 μ, since it was reasoned that with long hyphae the negative chemotropic stimulus would be much greater than with short ones. On the other hand, the positive chemotropic force should be exerted just as strongly on short hyphae as on long ones. In view of this, younger stages were tried, with results such as are set forth in table IV.

The combinations A and B correspond to those given previously, but C and D are new. D represents the control, for here conditions were made as much alike as possible in both upper and lower films. The turnip juice being everywhere practically the same in amount, no positive chemotropic force can be acting. If the number and length of the hyphae were also equal in both films, the turning on both sides should be no more pronounced in one direction than in

another.   It will be seen, however, that in every case the figures fall within the recognized experimental error.

TABLE IV

RESULTS IN RHIZOPUS NIGRICANS WITH TURNIP JUICE AND PLAIN AGAR; YOUNG STAGES OF GROWTH

Nature of films and location of spores	Number of preparations examined	Period of incubation in hours	Average length of hyphae in μ		Number of spores per sq. mm. of film surface	Total number of hyphae counted	Percentage of reaction
A Upper film: plain agar	2	6.75	40		37	108	+ 2*
	2	7.25	66		58	168	+18
Lower film: turnip juice agar+spores	2	8.00	111		56	192	+43
B Upper film: turnip juice agar	2	6.75	75		53	189	+94
	2	7.25	136		49	206	+99
Lower film: plain agar+spores	2	8.00	142		46	198	+94
C Upper film: turnip juice agar+spores	2	6.75	above 60	20	179	− 13
			below 51	15	118	+17
	2	7.25	above 80	23	158	− 8
			below 58	23	155	+33
Lower film: plain agar+spores	2	8.00	above 175	23	192	−30
			below 148	33	223	+46
D Upper film: turnip juice agar+spores	4	7.00	above 60	21	272	− 8
			below 74	27	292	+0.5
Lower film: turnip juice agar+spores	4	7.75	above 80	23	286	−6.5
			below 101	22	378	−2.0

* + and − signs indicate turning toward or away from the holes, that is, positive and negative reactions respectively.

The most marked turning is shown in *B*, where the hyphae while still very short (75 μ) show 94 per cent of reaction.   Here we have both chemotropic forces exerting a stimulus in the same direction. A most interesting contrast to this is shown in *A*, where the turning is toward a plain agar film without spores.   The turning is much less marked than in *B*, not in any case even half as great.   The hyphae, indeed, are shorter, but this is balanced by the fact that on the whole the spore number, and consequently the number of hyphae, is greater.   The result here, therefore, corresponds with the case of *A* among the older preparations described above, and the same observations apply here.   Also, as would be expected in

the young stage, the effect of the negative chemotropic force is very slight where the hyphae are only 40 μ long. Later, when more staling substance has developed with the growth of the hyphae, the percentage of turning toward the film free from staling substance is much increased; we have seen in the older preparations that when the hyphae attain a considerable length the turning may become nearly 100 per cent.

A consideration of C bears out our interpretation of A and B, for since both films here contain spores, the amount of staling substance is more or less equal throughout the preparation, and the force due to a negative chemotropic stimulus, therefore, is practically eliminated. Any reaction which occurs should be a positive chemotropic one, and, as the tables show, there is a considerable turning from the plain agar to the turnip juice agar. That the extent of this increases with the age of the preparation is not easily explained, though it is no doubt partly due to the fact that some of the hyphae naturally react more slowly than others.[7] On the other hand, the hyphae in the turnip juice agar show an evident repulsion from the plain agar. This repulsion also increases with the age of the preparation, although not so markedly. It may likewise be accounted for by a slower response of some of the hyphae, as well as by the fact that in this film the concentration of the turnip juice decreases, through diffusion, at first only in the immediate vicinity of the holes; later the decreasing concentration extends farther out from the holes and affects more hyphae.

EXPERIMENTS WITH CANE SUGAR.—Strengths of 2.5, 5, and 10 per cent cane sugar were tried, the cane sugar used being the ordinary commercial lump sugar. The following combinations were arranged in the experimentation with each percentage of sugar.

	A	B	C
Mica plate →	Plain agar / Agar+sugar +spores	Agar+sugar / Plain agar +spores	Agar+sugar +spores / Plain agar +spores

In 2.5 per cent cane sugar agar, the hyphae appeared very ill nourished, being slender and of slow growth. It seems reasonable

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[7] It may perhaps be caused also by the continual diffusion of the turnip juice farther and farther from the holes into the plain agar, thus acting on more and more hyphae.

to suppose that on this account they reacted weakly to chemotropic stimuli. As would be expected, the greatest turning was shown in *B*, where, when the hyphae were fairly long (200 μ) and sufficiently abundant, the percentage of reaction sometimes reached 40–50 per cent. That this turning was mainly due to a negative chemotropic reaction is apparent from what follows.

The experiments with 5 per cent cane sugar gave the best results, since this proportion seemed to supply the hyphae with a better amount of food, and also exerted a stronger positive chemotropic stimulus. Table V gives the results in condensed form.

TABLE V

RESULTS WITH 5 PER CENT CANE SUGAR

Combinations (as given in diagram above)	Number of preparations examined	Average length of hyphae in μ	Number of germ tubes per sq. mm. of film surface	Total number of hyphae counted	Percentage of reaction
A	3...........	116	17	162	+ 6
	4...........	133	25	282	+ 9
	4...........	155	19	195	+12
	3...........	189	23	185	+32
B	3...........	115	10	99	− 2
	3...........	139	14	150	+37
	3...........	162	12	184	+35
	4...........	197	14	229	+55
C	11........ { above 204	above 18	above 253	above −19
	{ below 191	below 5	below 280	below −10

Since it was clear from the experiments with turnip juice that the strength of the negative chemotropic stimulus was directly related to the length of the hyphae, the attempt was made to compare preparations containing hyphae of approximately equal length. Thus, the 4 lots of preparations in *A* and *B* roughly correspond. Unfortunately, the number of germ tubes averages much less in *B*, for many of the spores did not germinate, but if it were greater, assuming that it would be accompanied by an increased negative chemotropic stimulus, the final percentages in *B* would even be greater than they now are.

It is evident from the percentages that the hyphae in every stage of growth in *B* except the youngest are subjected to much

greater chemotropic stimuli than is the case in A. This agrees well with the experiments with turnip juice; in A we have the negative chemotropic stimulus working against the positive chemotropic stimulus, while in B we have both these forces working together.

The fact that no positive curvature appears in the early stage of B may be accounted for by the weak growth of the hyphae in non-nutrient agar. They thus do not react to the sugar diffusing from the upper film, nor have they yet produced sufficient staling substances to cause them to turn to the holes leading to the upper layer. But with the increasing diffusion of sugar they become in time better nourished, develop more of the staling substances, and react to this negative chemotropic stimulus as well as to the positive stimulus exerted by the sugar.

In C, although the average number of germ tubes per sq. mm. in the lower layer is given in the table as 5, a result of the poor germination in the plain agar, in this case among the 11 preparations holes were selected which had an approximately equal number of hyphae about them in both films. In order to have germ tubes of fairly equal length in both lower and upper films, the lower films were given a 4 hours' start before the upper films were added. The results, however, are not in line with what we have seen for the same experiment with turnip juice (C of table IV), for we would expect to find a positive reaction in the lower layer; but, as already observed, the spores germinate poorly and the hyphae grow slowly in the plain agar. The majority of them germinate only around the holes, where the sugar is diffusing through. With this sugar diffuse also staling substances from the more vigorously growing hyphae above, thus counteracting any stimulus which would otherwise be exerted on the hyphae of the lower layer by their own staling substance. The positive result in the similar case where turnip juice is used is probably due to the greater chemotropic activity of the turnip juice; and possibly also to a more rapid specific diffusion of the active substance of turnip juice as compared with cane sugar, as well as to the more vigorous growth of the lower layer hyphae which are nourished by the diffusing turnip juice.

With 10 per cent sugar, the results confirm the preceding.

EXPERIMENTS WITH GLUCOSE.—Glucose in strengths of 2.5, 5, and 10 per cent was also tried in the same way as described for cane sugar. The hyphae grew more vigorously in the glucose and reacted better. The results, in general, agree with those given for cane sugar.

MIYOSHI'S TEST BY DIRECT APPLICATION OF THE SUBSTANCES.—Among other tests for chemotropism, MIYOSHI (8) sowed spores in a film of 5 per cent gelatin on a glass slide, and when the germ tubes were still quite short, he placed a small amount of glucose at a given point in the gelatin. Diffusion commenced immediately, of course, and spread radially through the gelatin. With *Rhizopus*, he found that although some of the hyphae showed only more copious branching and an increase in thickness, others curved decidedly toward the center of diffusion. With *Penicillium*, however, no effect could be observed. On experimenting with other substances, he concluded that whenever no positive results were obtained, the failure was due to too rapid diffusion of the chemical substances.

Since this method was simple, and appeared practical, it was tried out extensively by the writer, using agar as well as gelatin films, and adding in some cases a small bit of solid glucose, in others, of cane sugar. Small pieces of turnip juice agar were tried also, these being fitted into corresponding cavities in the films. Neither *Rhizopus*, *Penicillium*, nor *Botrytis* gave any positive result, beyond the more copious branching and increased hyphal thickness mentioned by MIYOSHI. Even in the case of the turnip juice agar, no turning was evident on inspection with the microscope. Possibly, as MIYOSHI suggests, too rapid diffusion is the cause of the failure here. In any case, the mica plate method is far more definite and accurate.

Discussion

THE STALING SUBSTANCES.—From the foregoing it is clear that changes take place in a medium in which the fungus has grown, so that the medium then acts in a negatively chemotropic way toward the fungus. Whether these changes are due to the excretion of

katabolic products by the hyphae, or whether they are the result of chemical changes which these excreted products induce in the medium itself, it is not possible to state with certainty. In any case, vital processes of the fungus are primarily responsible.

Of these two possibilities, however, it is much more probable that the fungal excretions are themselves directly the cause of the negative chemotropic action, chiefly on account of the fact that *all* the media worked with produce a negative chemotropic reaction after the fungus has grown in them for a time. It is unlikely that with these various kinds of media the same repellent substances would invariably be formed by chemical action of the fungal excretions. In support of this view, it is also noteworthy that with *plain agar* the same repellent effect was evidenced as with other media.

That the staling substances are either of a volatile or of a thermolabile nature has already been conclusively demonstrated. BALLS (1) has also shown this, and has ascertained that they exert an inhibitory effect on growth.

RELATIVE VALUES OF POSITIVE AND NEGATIVE CHEMOTROPIC FORCES.—The great difference of various germ tubes in their capacity for reaction was very noticeable in the examination of the preparations. Thus, with two germ tubes at an early stage of growth, and at an equal distance from the hole, one would react markedly, while the other remained indifferent. At a later stage, however, when we may assume that the stimulus was greater, all of the germ tubes within the prescribed region about the hole might react.

Our knowledge of the relation between individual variation in sensitiveness of the germ tubes and the intensity of the acting stimulus is of course very vague,[8] but it seems safe to assume that a larger percentage of turning means a stronger stimulus. Also the rate of diffusion of the stimulating substances would, within limits, affect the number of germ tubes reacting in a given volume

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[8] It is probable that the variation curve of sensitiveness of the germ tubes is of the well known "normal" type, so that the percentage reacting is not directly proportional to the strength of the stimulus. TRÖNDLE (12) has shown that for geotropic sensitiveness the curve is of this type.

of material around the hole; and again, the time during which the stimulus had been acting (since a longer period gives greater opportunities for growth) would also affect the result. Bearing all this in mind, we nevertheless seem justified in assuming that under the conditions of any given experiment the percentage of turning gives some measure, although only a very approximate one, of the intensity of the stimulus or stimuli acting.

By an inspection of the results, especially as shown in tables IV and V, we can thus arrive at some idea of the relative intensity of the negative as compared with the positive chemotropic stimulus. For if we denote by n the percentage effect due to the negatively acting staling substances, and by p the percentage effect produced by the positive stimulus, we have in A of table IV, using the percentage of reaction in the oldest preparations, $n-p=43$; whereas in B we have $n+p=94$, taking the two oldest preparations here also. Since the hyphae in these two corresponding cases are of approximately equal length, and have therefore probably produced an essentially equal amount of staling substance, a comparison is legitimate. By eliminating the p's, we have $2n=137$, or $n=68.5$; p will then be 25.5. In other words, in the special condition of this experiment, the positive chemotropic stimulus exerted by the turnip juice has an effect of a little more than one-third that of the negative chemotropic stimulus. In a general way these figures are corroborated by the results given for C.

In the same way, applying this method to the results given in table V, we may arrange the relative values of the stimuli as shown in table VI.

With the exception of the youngest hyphae, where diffusion of the chemotropic substances has hardly begun, or at least the hyphae have not yet had time to react to it, the figures are surprisingly regular, the percentage of the reaction due to the positive chemotropic stimulus remaining fairly constant, as would be expected. On the other hand, the negative chemotropic stimulus constantly increases with the growth of the hyphae and the excretion of more and more of the negatively chemotropic substances.

In C of table V we should expect to find a confirmation of these deductions, as was shown by C of the turnip juice experiments

(table IV), but we have already seen why there is a discrepancy in the results for the lower film. On the other hand, the −19 per cent in the upper film is not greatly in excess of the amount representing the positive stimulus in A and B, for since the sugar in this case is in the upper film, the stimulus resulting from this would attract the hyphae away from the holes, and thus cause a negative figure.

TABLE VI

RELATIVE VALUES FOR POSITIVE AND NEGATIVE CHEMOTROPIC STIMULI IN CANE SUGAR EXPERIMENTS

Average length of hyphae	Nature of preparation	Percentage of total reaction	Percentage of reaction due to positive stimulus	Percentage of reaction due to negative stimulus
100–125	A B	(+ 6*) (− 2)	(− 4.0)	(+ 2.0)
125–150	A B	+ 9 +37	+14.0	+23.0
150–175	A B	+12 +35	+11.5	+23.5
175–200	A B	+32 +55	+11.5	+43.5
Very long†	A B	+49 +71	+11.0	+60.0

* The results in brackets all fall within the experimental error.
† These figures are taken from another series of experiments, not included in table V.

COMPARISON OF THE CHEMOTROPIC STIMULUS CAUSED BY CANE SUGAR AND BY TURNIP JUICE.—The cane sugar medium of 5 per cent strength, which seemed to be the proportion most favorable for growth, exerts, therefore, a comparatively small positive chemotropic force. Since it has been shown that when the turning is not very marked, the counting tends to be about 10 per cent too low, these numbers should probably be increased by about that percentage. But even if this be done, in comparison with it the stronger positive chemotropic force manifested by turnip juice is extremely striking and suggestive in relation to problems of parasitism, since it may indicate that plant juices in general evoke a fairly high positive chemotropic response.

Further work with plant juices is much to be desired.   These juices of course contain a large variety of substances, the combined effect of which is evidently (at least in turnip juice) much more powerful than that of cane sugar.   This is perhaps one of the reasons why Fulton, working with only simple substances like the sugars and various salts, was unable to demonstrate the existence of the very weak positive chemotropism which these substances may cause.   The fact should also be emphasized that the hyphae grew much more vigorously in turnip juice than in the sugars, and probably on that account reacted better as well as much earlier. It is reasonable to associate a stronger reaction with a healthier growth.

THE GRADIENT OF DIFFUSION IN ITS RELATION TO CHEMO-TROPISM.—Miyoshi (8) claimed that when hyphae are placed between two concentrations of a substance, the concentrations being kept constant, no chemotropic reaction occurred unless a definite ratio existed between the two concentrations.   Thus a fungus between 0.1 and 0.3 per cent of a sugar solution would not react; but with 0.1 and 1.0 per cent, a positive curvature occurred toward the 1 per cent.   Similarly a 0.5 per cent solution must have a 5 per cent solution as an antithesis, if a reaction is to ensue.   In other words, the conditions for a sufficient stimulus to produce a turning are in accordance with Weber's law.   In our own experiments, in the cases of the hyphae turning toward the holes, it is difficult to conceive how this law can apply.   The hyphae are so slender that the difference in concentration of the diffusing substance on opposite sides of a hypha, assuming that the latter is growing tangentially to the hole, could hardly be in the proportion of 10 to 1, given by Miyoshi as requisite for a reaction.   Jost (6) has voiced a similar criticism.   As a matter of fact, it is quite evident from our work that the hyphae must react to vastly smaller differences in concentration than this.   We must admit, however, that the external factors which bring about chemotropic reaction are still somewhat obscure.   Possibly there is a reaction to the direction of the diffusion current.

OSMOTROPISM.—It is possible that osmotropism, as suggested by Porodko (9), may play some small part in the reactions, but it cannot be an important factor, for if it were, we should get similar

results with solutions of equal osmotic pressure. Now, as regards the osmotic pressure of turnip juice, W. BROWN, working at this college, has determined that it is equal to that of a 14 per cent solution of cane sugar. It will be recalled that in the preparation of the turnip juice agar the turnip juice was diluted to half-strength, and its osmotic pressure must therefore be equal to about a 7 per cent solution of cane sugar. But our experiments show that even a 10 per cent solution of cane sugar is far less effective for causing a reaction than the turnip juice. It is clear, therefore, that osmotropism cannot be of great importance.

THE DISTRIBUTION OF A FUNGUS IN ITS HOST.—While the results given in this paper apply only to *Rhizopus nigricans*, there is abundant evidence from similar work carried on with *Penicillium* and *Botrytis* that the same conditions exist there also. It is quite likely that similar chemotropic reactions obtain in the majority of the fungi. If this be the case, it is possible that the distribution of a fungus in its host may depend mainly, not on a positive chemotropic reaction, but on the dominant negative chemotropic stimulus of its own staling products.

Summary

The following results apply to *Rhizopus nigricans* Ehrenb. in particular, but experimental evidence is at hand that the general principles involved apply also to *Botrytis cinerea* Pers. and *Penicillium* no. 24 Thom. Most of the data have been derived from work with two layers of medium separated by a perforated mica plate.

1. The fungus shows a marked negative chemotropic reaction to a medium in which it has been growing for some time.

2. The hypothesis brought forward by CLARK and FULTON, that this negative chemotropism is a reaction of the fungus toward its "staling substances," is conclusively substantiated by the following evidence: (*a*) the hyphae in one layer of medium turn away from another layer without spores if it contains their own staling products; (*b*) the hyphae always show a marked turning from the medium in which they are growing to any medium which is free of hyphae, no matter what the composition of that medium may be, unless the second medium contains the substances which exert a negative chemotropic stimulus; (*c*) when approximately

equal amounts of mycelium occur in two similar layers of medium, no turning of the hyphae from the one to the other results.

3. These staling substances are formed as a result of the vital activities of the fungus itself. They consist probably of excreted products of metabolism.

4. The staling substances appear to be either thermolabile or volatile, for boiling a solution containing them reduces markedly their negative chemotropic influence.

5. Positive chemotropism toward the substances tested (turnip juice, cane sugar, and glucose) also exists; but under ordinary conditions of growth this positive chemotropism is very much weaker than the negative chemotropism previously mentioned.

6. Turnip juice exerts a much stronger positive chemotropic stimulus than the other simple chemical substances tested, much stronger than, for example, 5 per cent cane sugar. This suggests the possibility that plant juices in general evoke a stronger positive chemotropic response than the simple chemical substances heretofore experimented with. The nature of the attractive substance or substances in turnip juice has not yet been determined.

7. It is impossible in the present stage of our knowledge to compare accurately the strength of two chemotropic stimuli; but, using the number of hyphae turning as a test of the relative action of the two stimuli, we certainly may conclude that in comparison with the stimulus exerted by negative chemotropism, that due to positive chemotropism is very much less. For example, in a given preparation with two layers of media separated by a perforated mica plate, and with the hyphae at a certain stage of growth, when 90–100 per cent of them are turning toward the holes from plain agar to turnip juice agar, the stimulus due to the positive chemotropic effect of the turnip juice has, very approximately, one-third of the effect of the stimulus due to the negative chemotropism from the staling products of the fungus itself. In other words, positive chemotropism is responsible at this stage and under these conditions for about one-quarter of the whole reaction. With the sugars the part played by positive chemotropism is very much less and is *easily overlooked*.

8. In contrast to the increase in strength of the negative chemotropic stimulus with the age of the preparation, the

attraction resulting from positive chemotropism remains fairly constant.

9. If the preceding conditions are true of fungi in general, it is probable that the distribution of a fungus in its host is influenced mainly, not by positive chemotropism, but by the dominant negative chemotropism due to its own staling products.

10. The part played by osmotropism in these reactions must, if any, be a very small one.

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